

# Effect of allopurinol, sulphasalazine, and vitamin C on aspirin induced gastroduodenal injury in human volunteers

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## Abstract

**Background**—The mechanisms of aspirin induced gastroduodenal injury are not fully understood. Aspirin induces the release of reactive oxygen metabolites in animal models, which may contribute to mucosal injury.

**Aims**—To investigate the effects of aspirin administered with placebo or antioxidants on gastric mucosal reactive oxygen metabolite release and gastroduodenal injury in human volunteers.

**Subjects**—Fourteen healthy volunteers participated in the study (seven male; mean age 27 years, range 20–40).

**Methods**—In a double blind, randomised, crossover study, volunteers received aspirin 900 mg twice daily and either placebo, allopurinol 100 mg twice daily, sulphasalazine 1 g twice daily or vitamin C 1 g twice daily for three days. Injury was assessed endoscopically and by quantifying mucosal reactive oxygen metabolite release by measuring chemiluminescence before and after each treatment. The effect on prostanoids was determined by measuring ex vivo antral prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis and serum thromboxane B<sub>2</sub> (TXB<sub>2</sub>).

**Results**—No drug reduced any parameter of gastric injury but vitamin C reduced duodenal injury assessed by Lanza score ( $p < 0.005$ ). Chemiluminescence increased after aspirin both with placebo ( $p < 0.05$ ) and vitamin C ( $p < 0.05$ ). Post-treatment chemiluminescence was lower in subjects taking allopurinol ( $p < 0.05$ ) or sulphasalazine ( $p < 0.005$ ) than in those taking placebo with aspirin.

**Conclusions**—In this study, aspirin induced gastric injury was associated with reactive oxygen metabolite release. This was reduced by sulphasalazine and allopurinol, although macroscopic injury was not affected. Vitamin C, however, was shown to have a previously unrecognised protective effect against aspirin induced duodenal injury.

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**Keywords:** allopurinol, sulphasalazine, vitamin C, aspirin, gastroduodenal injury.

mechanisms of injury are diverse and are not all fully understood. It seems, however, that aspirin damages the mucosal barrier, permitting the back diffusion of hydrogen ions, and inhibits prostaglandin synthesis by acetylating cyclooxygenase, thereby impairing mucosal blood flow, bicarbonate, and mucus secretion and epithelial cell hydrophobicity.<sup>2</sup>

Recent work suggests that neutrophil infiltration occurs early in non-steroidal anti-inflammatory drug (NSAID) induced gastric mucosal injury<sup>3</sup> and that neutrophil derived free oxygen radicals are probably contributory.<sup>4</sup> Free oxygen radicals have been shown to have an important role in several animal models of ischaemic injury,<sup>5–7</sup> which is known to occur after topical application of aspirin and hydrochloric acid to rat stomachs.<sup>8,9</sup> It seems probable, therefore, that reactive oxygen species released during inflammation and ischaemia may be a further mechanism of injury induced by aspirin.

Antioxidants have been successfully used in animals to reduce gastric injury induced by ischaemia,<sup>5–7</sup> aspirin,<sup>10,11</sup> and indomethacin.<sup>4,11,12</sup> One study found that allopurinol improved healing in patients presenting with bleeding from NSAID induced gastric erosions,<sup>13</sup> but effect on oxygen radical release was not investigated.

In this study, we have measured the reactive oxygen metabolite response to aspirin by measuring luminol and lucigenin amplified chemiluminescence from gastric mucosal biopsy specimens. In the presence of these oxidants, luminol and lucigenin form 3-aminophthalate and *N*-methylacridone, electronically excited compounds that emit light energy (chemiluminescence) on reverting to the ground state, which can be measured in a scintillation counter. We have investigated the effects of three antioxidants, allopurinol,

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## Modified Lanza scale of gastroduodenal mucosal injury

Lesion	Grade
No erythema	0
Erythema	1
No submucosal haemorrhage	0
1–4 Submucosal haemorrhages	1
5–10 Submucosal haemorrhages	2
11–20 Submucosal haemorrhages	3
Confluent haemorrhage	4
No erosion	0
1–4 Erosions	2
5–10 Erosions	3
11–20 Erosions	4
No ulcer	0
Ulcer	4

An ulcer is defined as a lesion causing a definite epithelial disruption in the mucosal surface and measuring greater than 3 mm in diameter.

Aspirin causes acute gastric mucosal injury in virtually all subjects<sup>1</sup> and longterm use is associated with gastric ulceration, and both gastric and duodenal ulcer bleeding.<sup>2</sup> The

sulphasalazine, and vitamin C, at known pharmacologically active doses, on the release of reactive oxygen metabolites, and related these effects to aspirin induced gastroduodenal injury quantified by endoscopy in human volunteers.

### Methods

Fourteen volunteers (seven male, mean age 27 years, range 20–40) participated in a double blind, randomised, crossover study consisting of four treatment phases: (a) aspirin 900 mg twice daily plus placebo; (b) aspirin 900 mg

twice daily plus sulphasalazine 1 g twice daily; (c) aspirin 900 mg twice daily plus allopurinol 100 mg twice daily; (d) aspirin 900 mg twice daily plus vitamin C 1 g twice daily.

A Latin square design was used to randomise the order of the treatment phases. Each phase was separated by a washout period of at least one week. Subjects took their medication 30 minutes before taking the aspirin and 60 minutes before meal times. The first dose was taken at 7 am on day 1 and the last dose at 7 am on day 3, prior to endoscopy at 8 am. Subjects were asked to avoid other medications, alcohol, and spicy foods for the duration of the study. Pre-study screening ensured that all volunteers had a normal physical examination, full blood count, coagulation studies, and biochemistry tests and that women had a negative pregnancy test. Approval from the University of Nottingham Ethics Committee was obtained and written informed consent was provided by all volunteers.

### Endoscopy

Unsedated endoscopy was performed by the same endoscopist in all subjects before and after each treatment phase. The presence of erythema and the number of erosions (haemorrhagic and non-haemorrhagic), ulcers, intramucosal haemorrhages, and surface flecks of blood were recorded for oesophagus, gastric body, antrum, and duodenum. Injury was also quantified using a modified Lanza scale (Table).<sup>14</sup>

### Chemiluminescence

Biopsy specimens were taken from the antrum on the greater curve for measurement of both luminol and lucigenin amplified chemiluminescence. Each specimen was immediately washed in phosphate buffered saline containing 5 mmol/l glucose (pH 7.4) and transferred to a dark adapted scintillation vial containing 1 ml luminol or lucigenin (both at 300  $\mu$ mol/l). All solutions were pre-oxygenated. Chemiluminescence was measured over five minutes in a LKB Wallac  $\beta$  scintillation counter (model 1214) operating in out of coincidence mode. Specimens were then carefully blotted dry and weighed and chemiluminescence expressed as counts per minute per gram of tissue (cpm/g).

### Prostanoids

Ex vivo synthesis of prostaglandin  $E_2$  ( $PGE_2$ ) by antral mucosal biopsy specimens taken from the greater curve was measured by radioimmunoassay as previously described.<sup>15</sup> Samples were washed in 1 ml TRIS saline, vortex mixed, and centrifuged at high speed twice, after which the supernatant was discarded. On the third occasion, 0.3 ml TRIS saline was added to each specimen followed again by vortex mixing and centrifugation. The supernatant was removed and frozen at  $-40^\circ\text{C}$ . Blood was taken immediately after each endoscopy and allowed to clot for 30 minutes at  $37^\circ\text{C}$ . Thromboxane  $B_2$  ( $TXB_2$ )

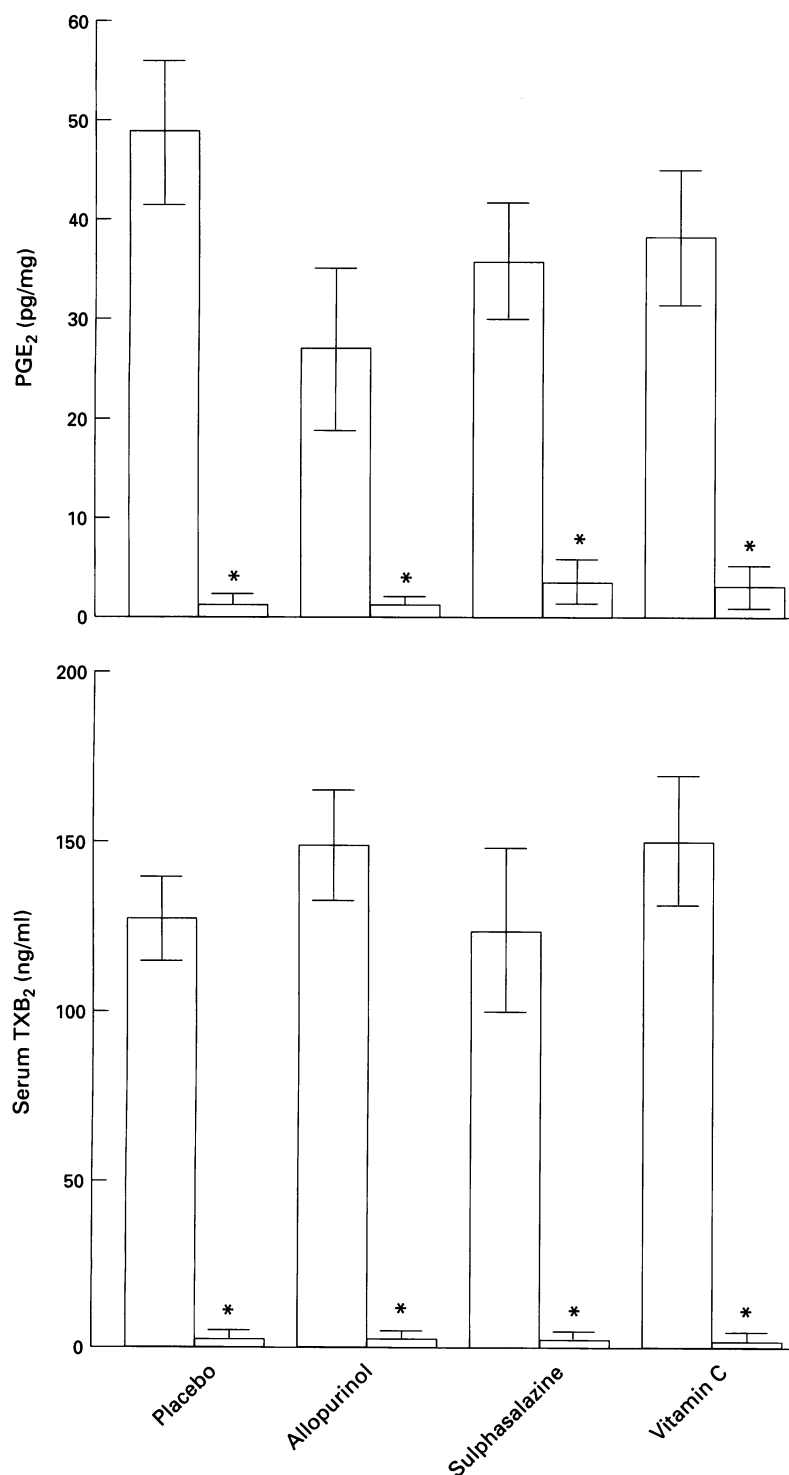


Figure 1: Prostanoid values before and after three days' treatment with aspirin and placebo or antioxidant. Values are mean (SEM). \* $p < 0.001$  compared with pre-treatment value.

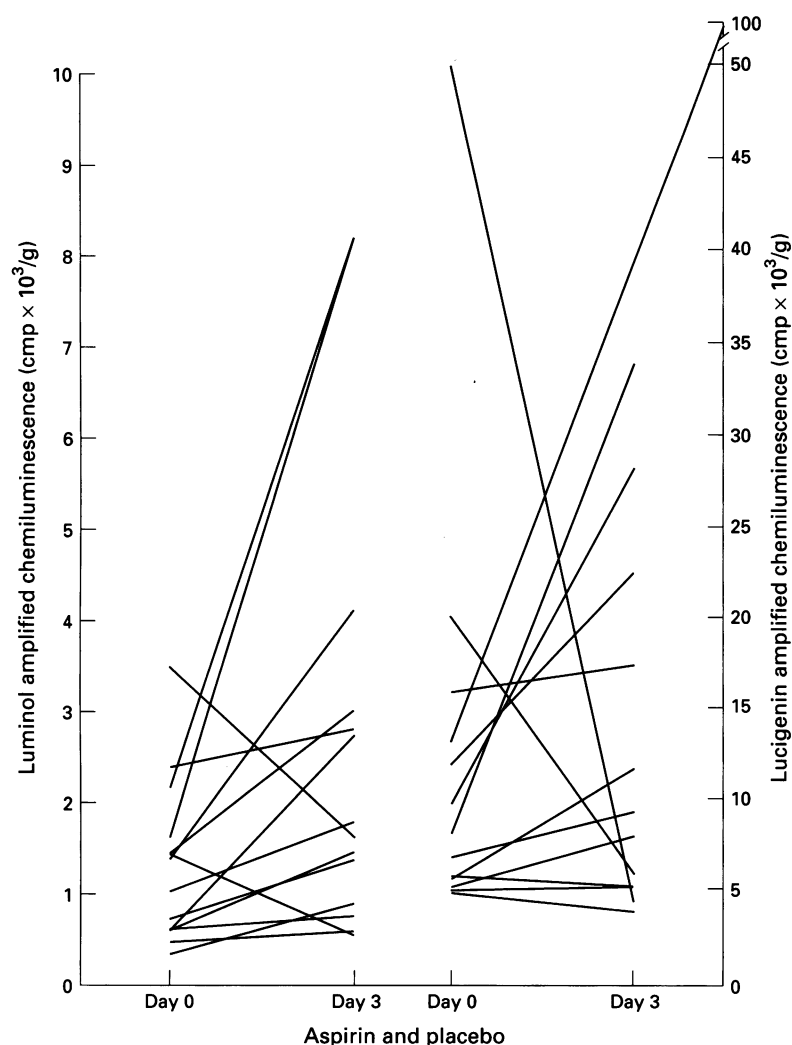


Figure 2: Luminol and lucigenin amplified antral biopsy chemiluminescence from 14 subjects before (day 0) and after (day 3) three days' treatment with aspirin and placebo.

was measured in the separated serum by radioimmunoassay.<sup>15</sup>

#### Statistics

The Wilcoxon rank sum test was used for subgroup analysis of paired data if an effect was observed following Friedman's analysis of variance. The Mann-Whitney test was used for unpaired data. The data are shown as mean (SEM).

#### Results

##### Prostanoids

Mean (SEM) baseline  $\text{PGE}_2$  synthesis was  $48.8$  ( $7.3$ )  $\text{pg/mg}$ . This fell to  $1.4$  ( $1.8$ )  $\text{pg/mg}$  after aspirin ( $p < 0.001$ ). Mean baseline serum  $\text{TXB}_2$  was  $12.7$  ( $1.3$ )  $\text{ng/100 } \mu\text{l}$ , and after aspirin and placebo values were beneath the limits of the assay ( $< 0.2$   $\text{ng/100 } \mu\text{l}$ ,  $p < 0.001$ ) in most cases. There were no differences in tissue  $\text{PGE}_2$  synthesis or serum  $\text{TXB}_2$  after aspirin in the presence of allopurinol, sulphasalazine or vitamin C (Fig 1).

##### Chemiluminescence

Figure 2 shows the effect of aspirin and placebo

on luminol and lucigenin amplified chemiluminescence from gastric biopsy specimens. After aspirin and placebo, luminol amplified chemiluminescence rose in 12 of 14 subjects from a mean baseline value of  $1.3 \times 10^6$  ( $2.4 \times 10^5$ )  $\text{cpm/g}$  to  $2.7 \times 10^6$  ( $6.8 \times 10^5$ )  $\text{cpm/g}$  ( $p < 0.05$ ). There was also a significant rise after aspirin and vitamin C, from  $1.3 \times 10^6$  ( $1.9 \times 10^5$ )  $\text{cpm/g}$  to  $2.5 \times 10^6$  ( $6.1 \times 10^5$ )  $\text{cpm/g}$  ( $p < 0.05$ , Fig 3). Coadministration of allopurinol with aspirin abolished the increase induced by aspirin, as the mean value fell from the baseline of  $1.3 \times 10^6$  ( $2.7 \times 10^5$ )  $\text{cpm/g}$  to  $1.2 \times 10^6$  ( $2.6 \times 10^5$ )  $\text{cpm/g}$  after treatment ( $p = 0.7$ , NS). Mean values also fell marginally after aspirin with sulphasalazine from a pretreatment value of  $1.2 \times 10^6$  ( $2.7 \times 10^5$ )  $\text{cpm/g}$  to  $8.3 \times 10^5$  ( $1.3 \times 10^5$ )  $\text{cpm/g}$  ( $p = 0.5$ , NS). A comparison of mean 3 day values show that both allopurinol ( $p < 0.05$ ) and sulphasalazine ( $p < 0.005$ ) significantly reduced luminol amplified chemiluminescence compared with aspirin and placebo (Fig 3).

Lucigenin amplified chemiluminescence increased in nine of 14 subjects after aspirin and placebo. The mean baseline value of  $1.3 \times 10^7$  ( $3.7 \times 10^6$ )  $\text{cpm/g}$  increased to  $2.0 \times 10^7$  ( $7.8 \times 10^6$ )  $\text{cpm/g}$  after treatment, but this did not reach statistical significance ( $p = 0.2$ , Fig 2). Aspirin given in conjunction with vitamin C produced an insignificant reduction in mean lucigenin amplified chemiluminescence value, which fell from a baseline of  $1.3 \times 10^7$  ( $3.1 \times 10^6$ )  $\text{cpm/g}$  to  $1.1 \times 10^7$  ( $2.5 \times 10^6$ )  $\text{cpm/g}$  (Fig 3). Reductions in mean values occurred after aspirin with allopurinol, from a baseline of  $1.5 \times 10^7$  ( $9.2 \times 10^6$ )  $\text{cpm/g}$  to  $7.5 \times 10^6$  ( $1.5 \times 10^6$ )  $\text{cpm/g}$  ( $p = 0.6$ , NS) and after aspirin and sulphasalazine, from  $9.3 \times 10^6$  ( $3.3 \times 10^6$ )  $\text{cpm/g}$  to  $7.7 \times 10^6$  ( $1.3 \times 10^6$ )  $\text{cpm/g}$  ( $p = 0.2$ , NS).

##### Mucosal injury

No subject had erosions or ulcers in any area at baseline investigation (Lanza grade 0–1). Oesophagitis was present in two subjects after a total of three treatment periods, but no oesophageal injury was sustained by the remaining 12 subjects throughout the study. The median Lanza score in the gastric body was 2 (range 1–4) after treatment with aspirin and placebo, and 2.5 (0–4) after each of the other treatment phases (aspirin with either allopurinol, sulphasalazine or vitamin C). After treatment with aspirin and placebo the median Lanza grade in the antrum was 3 (2–4), compared with 2 (0–4) after cotreatment with either allopurinol or sulphasalazine and 3 (0–4) with vitamin C. The combined scores (for antrum and body) were 4 (2–4) after aspirin and placebo, 4 (1–4) after aspirin and either allopurinol or vitamin C, and 3.5 (0–4) after coadministration with sulphasalazine (Fig 4). Duodenal injury reached a median value of grade 2 (0–4) (Fig 5) after aspirin and placebo and grade 1 (0–4) after aspirin with each of the other three drugs. Vitamin C significantly reduced the aspirin induced injury in the duodenum as measured by the Lanza score in the duodenum compared with placebo ( $p < 0.005$ ).

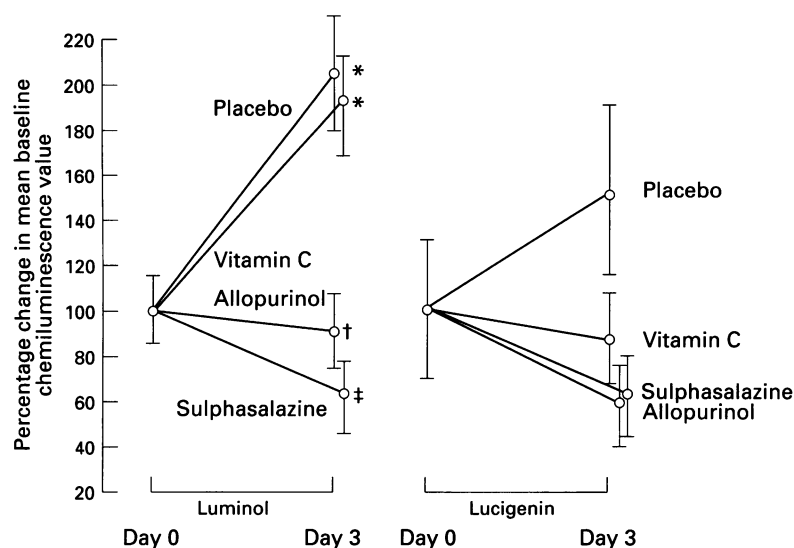


Figure 3: Luminol and lucigenin amplified antral biopsy chemiluminescence before (day 0) and after (day 3) three days' treatment with aspirin and placebo or antioxidant. Values are mean (SEM) and are represented as a percentage of the baseline (pre-treatment) value. \* $p < 0.05$  compared with baseline value, † $p < 0.05$  and ‡ $p < 0.005$  compared with placebo day 3 value.

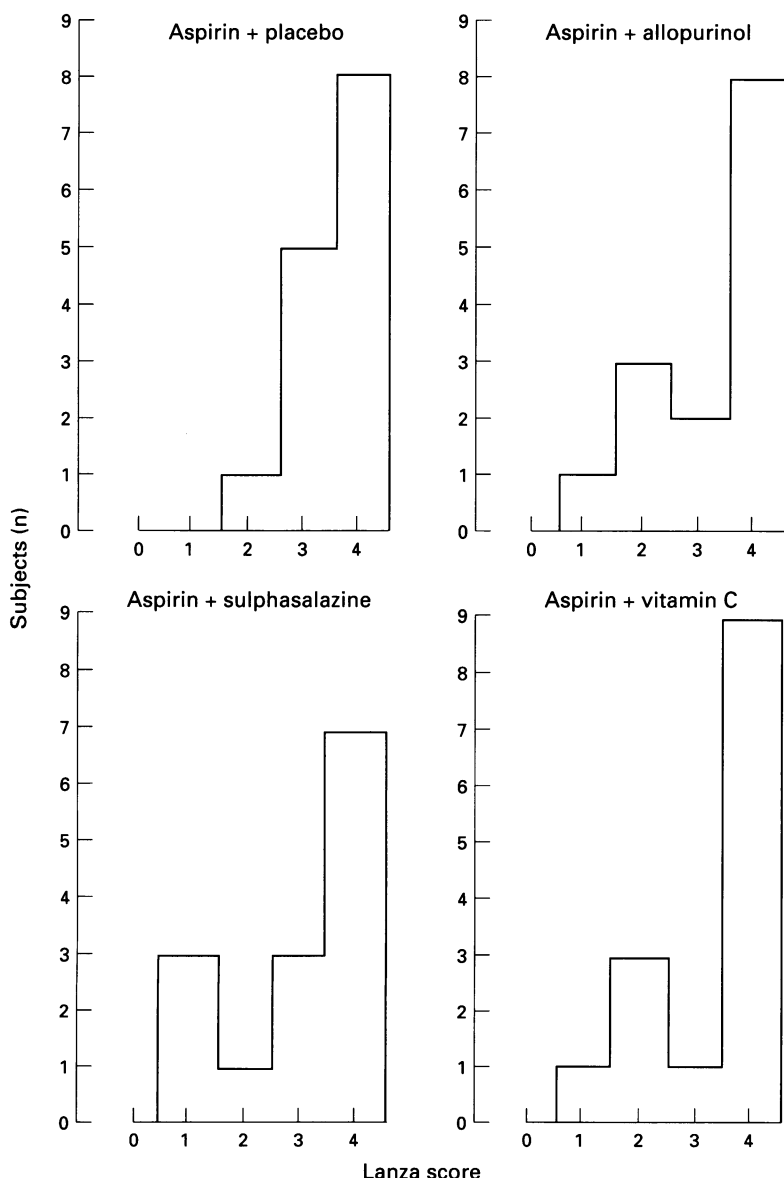


Figure 4: Frequency distribution graphs for Lanza grade in the stomach (antrum and body combined) after each treatment regimen.

Duodenal ulcers developed in no subject given vitamin C, but did in two subjects in each of the placebo and allopurinol groups and in three of the sulphasalazine group. All ulcers, defined as lesions causing definite epithelial disruption in the mucosal surface and measuring at least 3 mm in diameter, were superficial and healed spontaneously and completely by the time of the next endoscopy.

## Discussion

Our study shows that aspirin induced gastric mucosal injury in humans is associated with an increase in the release of reactive oxygen metabolites, which may themselves contribute to mucosal injury.<sup>4 11 12</sup> In our study, coadministration of either allopurinol or sulphasalazine with aspirin was associated with a reduction in reactive oxygen metabolite release, but neither of these drugs protected against gastroduodenal injury. However, vitamin C seemed to have a hitherto unrecognised protective effect against aspirin induced duodenal injury despite failing to show any antioxidant activity in the stomach. Our findings are in keeping with previous studies in rats and humans that show increased synthesis of reactive oxygen metabolites in NSAID induced damage,<sup>4 10-13</sup> but do not substantiate data suggesting that antioxidants may protect against gastric mucosal injury.<sup>4 11-13</sup>

Aspirin induced an increase in luminol, but not lucigenin reactive free oxygen radicals. Luminol predominantly measures products of the neutrophil derived myeloperoxidase pathway whereas lucigenin is more specific for the superoxide.<sup>16 17</sup> The enhanced luminol amplified chemiluminescence thus suggests that myeloperoxidase is an important source of reactive oxygen species, which would be consistent with evidence that neutrophil infiltration occurs in NSAID induced gastric mucosal injury.<sup>3</sup> The absence of an increase in lucigenin amplified chemiluminescence in response to aspirin might reflect instability of the superoxide in the acid environment of the stomach. Alternatively, although both luminophores react with extracellular oxidants, only luminol is thought to penetrate the cell,<sup>18</sup> perhaps suggesting that the source of increased chemiluminescence is predominantly intracellular. It is impossible to say, however, if the very weak trend ( $p = 0.2$ ) toward an increase in lucigenin amplified chemiluminescence was real or not.

In addition to stimulated neutrophils, a further source of reactive oxygen species in the stomach might be endothelial xanthine oxidase, formed by conversion from the dehydrogenase after an ischaemic injury, and capable of converting molecular oxygen to the superoxide after reperfusion. Studies in dogs have shown that topical aspirin induces pallor, ulceration, and haemorrhage and is associated with a pronounced reduction in blood flow at the site of damage.<sup>8 9</sup> Kitahora and Guth observed that aspirin in hydrochloric acid topically applied in rats induced thrombi in the mucosal microvessels and a cessation of mucosal blood flow.<sup>19</sup> This may be of potential

clinical significance, as free oxygen radicals clearly contribute to gastric damage in animal models of ischaemia reperfusion, and may be reduced by allopurinol, a xanthine oxidase inhibitor.<sup>5-7</sup> Indeed one study does suggest that allopurinol can protect against indomethacin induced injury in rats.<sup>20</sup> It is known that indomethacin produces a global reduction in gastric blood flow in humans within 24 hours of administration.<sup>21 22</sup> Whether the effect of NSAIDs on human gastric blood flow, either focally or globally, is sufficient to induce oxidation of xanthine dehydrogenase is not known. Our data indirectly support the hypothesis that local ischaemia produced by aspirin in humans might induce the release of reactive oxygen metabolites produced by xanthine oxidase as allopurinol substantially reduced the increase in aspirin induced chemiluminescence. We were unable to show, however, that this was associated with significant mucosal protection.

Salicylate compounds have long been recognised as having a protective effect against NSAID induced gastric injury, although the underlying mechanism has been obscure. Sodium salicylate was shown to protect against aspirin and indomethacin induced gastric ulceration in rats<sup>23 24</sup> and gastrointestinal bleeding in humans.<sup>25</sup> A decade later, evidence emerged suggesting that sulphasalazine possessed similar properties. When given parenterally to rats it was found to have a protective effect against various models of gastric injury, including alcohol and phenylbutazone,<sup>26</sup> but

not indomethacin<sup>27</sup> (although it did prevent small bowel ulceration induced by indomethacin).<sup>28</sup> The mechanism for this antiulcer activity was not fully explained, but it was thought to be independent of prostaglandin formation. It was suggested in the earlier studies that the protective effect of sodium salicylate was in part due to competition for the active cyclooxygenase catalytic site<sup>29</sup> or a reduction in drug absorption.<sup>30</sup> There was no evidence of reduced drug absorption or a reversal of cyclooxygenase inhibition in this study as sulphasalazine failed to change the aspirin induced suppression of prostaglandin synthesis.

Since then, much evidence has accumulated that sulphasalazine has antioxidant activity, in addition to its various other anti-inflammatory properties. It reacts with free oxygen radicals in the colon of patients with inflammatory bowel disease<sup>31</sup> and has been shown to inhibit myeloperoxidase and superoxide release in an animal model<sup>32</sup> and stimulated human neutrophils<sup>33-37</sup> respectively. This is the first study to show that sulphasalazine acts as an antioxidant in the human stomach, a mechanism that may partly explain the previous identified gastric protective effects of salicylates. Aspirin may induce injury by several mechanisms, however, and the reduction of free oxygen radicals was insufficient to reduce visible gastroduodenal injury in our study.

We used vitamin C in this study for two reasons: firstly because it is a free oxygen radical scavenger, and secondly because it has been suggested that the gastric mucosa is more prone to injury (particularly by aspirin) in the presence of diminished leucocyte ascorbic acid concentrations.<sup>38 40</sup> Reduced values are found in patients with rheumatoid arthritis,<sup>41 42</sup> but these fall still further in those taking regular aspirin.<sup>41</sup> Moreover, low values have been found in patients with peptic ulcer<sup>38 39</sup> and gastrointestinal haemorrhage,<sup>39</sup> particularly in those with bleeding precipitated by aspirin. Russell and Goldberg confirmed an increased risk of gastric bleeding in guinea pigs given aspirin when receiving a scorbutogenic diet.<sup>40</sup>

Vitamin C supplements, however, had no effect on aspirin induced gastric release of reactive oxygen metabolites and did not protect against gastric injury in our study. Ascorbate is a hydrophilic compound that cannot scavenge lipophilic radicals within lipid membranes,<sup>43</sup> a property that might prevent it reacting in the lumen with radicals induced by aspirin. It may still have a role as an antioxidant following absorption, however, as it is known to be concentrated in the gastric epithelium<sup>44</sup> and reaches high concentrations after oral supplementation.<sup>42 45-48</sup> Possibly the most important mode of absorption is by an active, sodium dependent transport mechanism from the small bowel.<sup>49 50</sup> There is, however, some evidence that suggests that aspirin impairs sodium dependent transport mechanisms.<sup>51</sup> Furthermore, in one study the coadministration of vitamin C with aspirin seemed to impair absorption,<sup>42</sup> although this has been disputed.<sup>45 46</sup> In all three studies, however, aspirin diminished uptake of ascorbate into

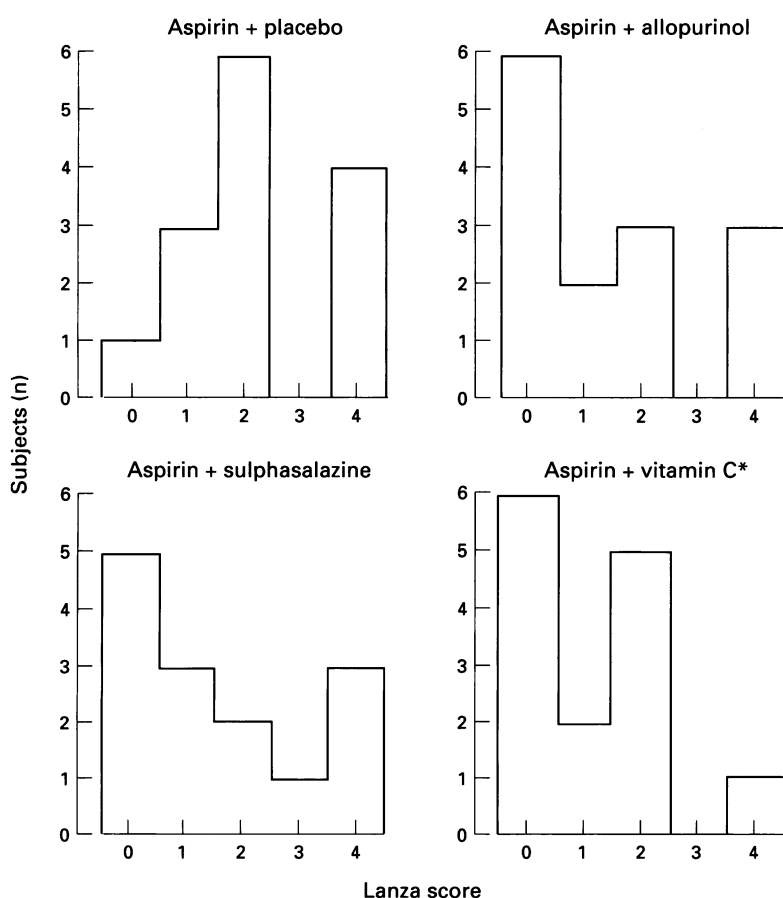


Figure 5: Frequency distribution graphs for Lanza grade in the duodenum after each treatment regimen. \*p<0.005 by comparison with aspirin and placebo.

leucocytes,<sup>42 45 46</sup> which might permit the unopposed synthesis and release of free oxygen radicals by neutrophils activated after NSAID induced gastric mucosal injury.<sup>4</sup>

Changes in absorption may explain the otherwise paradoxical protection of the duodenum by vitamin C. The pKa of ascorbic acid is 4.1.<sup>39</sup> Hence at normal gastric pH, vitamin C is present predominantly as ascorbic acid, which will tend to be oxidised to dehydroascorbic acid as the pH rises in the duodenum. There is some evidence that vitamin C is more readily absorbed as dehydroascorbic acid.<sup>52 53</sup> This may partly be because it is less ionised at physiological pH and is promptly reduced to ascorbic acid and thereby trapped within the epithelium.<sup>52 53</sup> It is possible that this mechanism allows sufficient absorption of vitamin C to enable it to act as an antioxidant within the duodenal mucosa.

Vitamin C has other actions that may be relevant to mucosal repair and therefore must be considered. The role in wound healing through the synthesis of collagen, the formation of intercellular matrix, and angiogenesis is well established.<sup>54</sup> Furthermore, ascorbate is an important reductant in the  $\alpha$ -amidation of peptide hormones, including gastrin. Wound healing improves once vitamin C deficiency is corrected<sup>55</sup> and ascorbic acid has been used with some success in the treatment of leg ulcers<sup>56</sup> and pressure sores.<sup>48</sup> This may represent another mechanism by which vitamin C encourages mucosal healing in the duodenum.

Aspirin induces gastroduodenal injury by a variety of mechanisms. We have shown that aspirin increases the production of gastric mucosal reactive oxygen metabolites, which are likely to act as a further source of injury. Our data suggest that oxygen radical release may occur after neutrophil activation and an ischaemia-reperfusion injury, both of which mechanisms have been implicated in NSAID induced gastric mucosal damage. Antioxidants did reduce antral oxygen radical release, but failed to provide gastric mucosal protection, reflecting the multifactorial nature of aspirin induced injury. Vitamin C seemed to have a protective effect on aspirin induced duodenal injury, the mechanism for which requires further elucidation. Preferential absorption in the duodenum may enable it to accumulate and act as an antioxidant within the mucosa, but it has other actions in tissue repair, which may be equally important.

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